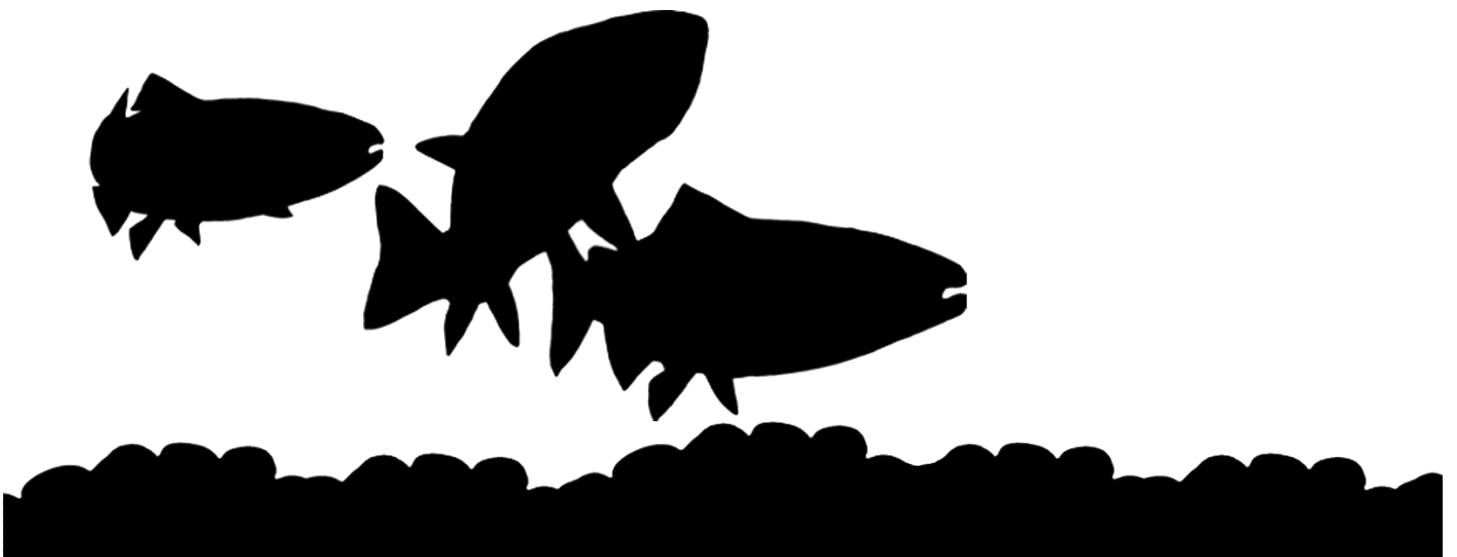# Deformity Prevalence and Meristic Characteristics in Atlantic salmon: *The Effect of Ploidy, Incubation Temperature and Hybridization*

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Master of Science Thesis
2013

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2013

Deformity Prevalence and Meristic Characteristics in Atlantic salmon: The effect of Ploidy, Incubation Temperature and Hybridization

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Acknowledgements

I first and foremost would like to thank Havforskningsinstituttet for the amazing opportunity to take part in this experiment. Per Gunnar Fjelldal and Tom Hansen were of unbelievable help throughout this entire project and working with them was a privilege. The trips to the Matre research station were something special that I will remember for a long time.

I would also like to thank Asbjørn Vøllestad. Although my experiment was not in Oslo, or me for that matter, Asbjørn was always willing to help and gave me some very valuable advice through the writing part of my project. He is always quick to answer emails and help with corrections and always seem to have time to meet and guide me through this project. Thank you very much and I feel very privileged to have had you as my supervisor.

To my family: You guys have helped me so much throughout these two years it's hard to even begin. Your love and support has blown my mind and I couldn't have done it without you. Thank you so much and I love you guys very much.

Thank you to my all my Norwegian pals. You took me in and made Norway feel like a second home. I have met some awesome people over the past two years who has made Norway extremely enjoyable. I would especially like to thank Marius and Kjersti for the countless hours of studying as well as the great times outside of school.

Last but defiantly not least, I would like to thank Kris. Your love and support has been amazing throughout these two years and I couldn't be more grateful. You are an amazing person and I couldn't be luckier. Thank you so much and I love you very much!

Contents

Abstract

Intense salmon farming regimes in Norway have resulted in hundreds of thousands of salmon escaping each year. These domesticated salmon have been selectively bred for generations and therefore have the potential to genetically infiltrate wild population's causing gene flow, out breeding depression and ultimately a decline in stocks. Triploidization has become a popular method for inducing sterility into large batches of salmon. This study investigated the effects of triploidization on Atlantic salmon and Atlantic salmon x Arctic char hybrid. Part one of the experiment investigated the effect of incubation temperature on triploid salmon with regards to vertebra number and deformity prevalence, while part two investigated the effect of triploidization on Atlantic salmon x Arctic char hybrid with regards to meristic characteristics. The goal of the study is to help identify rearing conditions for triploid Atlantic salmon that would reduce the presence of deformities and determine the effect of triploidization on salmonid hybrid morphology. The results of this study suggest that incubating triploid embryos at lower temperatures will reduce the prevalence of vertebrae deformities. I also found that hybrid morphology of triploid fish is expressed in a non-linear fashion with regards to genetic contribution. This study assists in the understanding of triploidization in hopes that one day the procedure will be a standard in European salmon farming regimes.

Introduction

The salmon aquaculture industry in Norway is one of the largest producers of Atlantic salmon (*Salmo salar*) in the world. It is the 5th largest export for Norway behind oil and heavy metals and accumulates to nearly 15.3 billion Norwegian kroner each year (FAO, 2010). In 2006, nearly 450 million Atlantic salmon were produced and of that 700,000 escaped into the wild (FAO, 2010). Not only are these escapees a massive loss in production but poses a serious threat to wild populations of fish.

The threat of escaping farmed salmon is one of the largest environmental challenges for the salmon aquaculture industry. Growing concern for environmental welfare and reduced wild salmon populations has put pressure on the aquaculture industry to eradicate this problem. Domesticated fish have been selectively bred for generations which have caused them to acquire traits that are advantageous to the industry but not suited for natural environments (McGinnity et al., 1997). Genetically similar populations of animals are advantageous when creating a product for a consumer but from a biological viewpoint renders the population susceptible to disease, pathogens or environmental change (McGinnity et al., 2003). Thus, it is important for wild populations of fish to maintain a level of genetic variability in order to have the ability to respond to changing environmental conditions (Jacq et al., 2011).

When domesticated fish escape and breed with wild populations, genetic dilution occurs which puts wild populations at risk of gene flow. Gene flow is the micro evolution of a population due to the transfer of alleles or genes between populations (Bohonak 1999). Domesticated strains of salmon used in Norway originated from wild stocks and therefore contain the same gene pool as wild populations (Jacq et al., 2011). However, over generations of selective breeding the allele frequency of desired traits has increased and therefore when escapees breed they have the potential to change the genetic structure of wild populations. This may cause wild populations to experience outbreeding depression or reduced fitness due to the loss of adaptation (McGinnity et al., 2003). Attempts at solving this problem via closed net systems or onshore farming have either failed or been too expensive for feasible use. Therefore major funding has been directed towards research involving triploid fish in hopes of implementation into European farming regimes.

At the moment, there are only two acceptable ways of sterilizing fish, triploidization and hybridization (Tiwary et al., 2005; Bartley et al., 2001). There are other methods that involve chemicals or gene manipulation but the outcome of these would render the fish unacceptable for a consumer market. The method of triploidization has become a refined and reliable tool to ensure sterility in large batches of fish. The method was pioneered by Benfey et al. (1984) and involves the manipulation of the cell shortly after fertilization to retain the second polar body, a procedure which renders the embryo triploid (Benfey 1984). The original method involved heat shocks but over time the procedure has evolved and today's method relies on hydrostatic pressure to be applied at specific time intervals following fertilization (Maxime 2008; Fjelldal and Hansen 2010). The hydrostatic pressure interferes with the spindle fibers, which allow for the egg to retain the second polar body during meiotic division (Tiwary et al., 2004) the result is an egg containing two sets of maternal chromosomes and one paternal.

Once development begins each cell in the triploid fish will have a third set of chromosomes. The acquisition of a third set of chromosomes causes the nucleus of each cell to be larger in triploids than that of diploids. Interestingly, the overall size of the fish is unaffected and triploid fish are remarkably similar in size to that of their diploid conspecifics (Maxime 2008; Benfey 1999). This is due to the fact that triploid fish have an overall lower cell count when compared to diploids (Tiwary et al., 2005; Benfey 1999). Larger cell sizes can be seen visually when looking at the erythrocytes of triploid fish and is consequently used as a ploidy recognition tool (Benfey et al., 1984).

Morphological features of diploids and triploids appear to be remarkably similar (Maxime 2008; Tiwary et al., 2005; Benfey 1999). In regards to Atlantic salmon the major morphological difference reported between diploids and triploids is the frequent occurrence of skeletal deformities (Sutterlin et al., 1987; McGeachy et al., 1996; Wargelius et al., 2005; Witten et al., 2009; Fjelldal and Hansen., 2010). The most common of these deformities are malformations in the vertebral column including elongation, compression and fusion (Fjelldal and Hansen 2010; Witten et al., 2009). Due to production loss and consumer acceptance these skeletal deformities have proven to be a major bottleneck in the implementation of triploid fish into the aquaculture industry. Therefore it is important to elucidate the

conditions in which deformities manifest in order for triploidization to become a formidable procedure for fish domestication.

Temperature is considered the most influencing abiotic factor with regards to growth and development in ectotherms (Pepin 1991). When it comes to Atlantic salmon, thermal conditions experienced by embryos during incubation have a large effect on the natural growth variation (Elliot & Hurley 2003). Embryogenesis is an important time in development where organs and external features begin to take form (Fowler 1970) and different incubation temperatures have shown to affect the vertebral column of salmon species (McDowall 2008; Ando et al., 2011). The sensitivity of vertebrae development to varying incubation temperature and the genetic nature of triploids may indicate different responses to varying incubation temperatures in triploid salmon than in diploid salmon.

Thermal reaction norms are defined as the profile of phenotypes produced by a given genotype across an environmental gradient (Griffiths et al., 2000). They are commonly studied to understand the way species react to changing abiotic factors. In Atlantic salmon a negative reaction norm is commonly observed with regards to vertebra number across a temperature gradient. These reaction norms include progressively decreasing vertebra number with increasing incubation temperature (Ando et al., 2011). It has been suggested that studying these reaction norms would give insight to optimal conditions for proper development (Ando et al., 2011). Therefore it would be advantageous to investigate the effect of varying incubation temperature on triploid salmon and the possible effect it has on deformity prevalence. Doing so may indicate rearing conditions that would be better suited for triploid Atlantic salmon.

The second method of producing sterile fish is by hybridization with closely related species. Hybridization within salmonids, in many cases, yields viable organisms that are unable to reproduce due to problems with gonad development and chromosome paring (Bartley et al., 2001). In addition, intraspecific hybrids have the potential of combining desirable characteristics of two species (Bartley et al., 2001). Many fish species are farmed for specific advantages ranging from disease resistances to flesh color or taste. The combination of desirable characteristics could open the door for new possibilities of fish for domestication which could prove beneficial for both the producer and consumer. There are many examples

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used throughout the industry which have taken advantage of the compatibility of species. A cross between white bass (*Morone chrysops*) and the striped bass (*M. saxatili*) produced offspring which under commercially cultured conditions grow faster and react better to tanks and cages than the parental species (Hallerman 1994). Increased disease resistance has been shown with crosses between rainbow trout (*Oncorhynchus mykiss*) and char (*Salvelinus sp.*) (Dorson et al., 1991). Hybrids offer an interesting potential within aquaculture industry and becomes even more attractive when the method of triploidization is applied.

As previously mentioned, a triploid organism will have two sets of maternal chromosomes and one set of paternal chromosomes. Therefore, due to genetic contribution, it would be assumed that the morphological expression of a triploid hybrid will lean more towards that of the maternal species. This idea of morphological expression due to the genomic contribution is known at the Genetic dosage effect (GDE)(Kierzkowski et al., 2011). The GDE has been studied thoroughly in hybrid plants, which tend to show a linear relationship between morphological expression and genomic contribution (Chen and Ni 2006). However, when studying organisms such as hybrid frogs a mosaic pattern of expression is observed, in that morphology of the hybrid varies in similarity between the parental species (Kierzkowski et al., 2011). GDE has not been studied in hybrids between salmon and other salmonids but an understanding of the effect could prove advantageous to the aquaculture industry. Acquiring traits via hybridization and amplifying desired ones via triploidization, as well as securing sterility, could prove a unique way of discovering new sterile candidates for fish domestication.

The following study was a two part experiment. In part one, three different temperatures were used to incubate triploid and diploid Atlantic salmon eggs. The projects objective was to investigate what effect of different incubation temperatures had on diploid and triploid salmon and concurrently determine the potential effects it has on vertebra number and deformity prevalence. Thermal growth reaction norms were investigated for both vertebra number and vertebra size. The overall goal of this experiment was to better understand triploid Atlantic salmon and the rearing conditions they may require for optimal development.

In part two, I investigated a cross between Atlantic salmon and Arctic char (*Salvelinus alpinus)*. The char-salmon hybrid was examined for meristic characteristics in order to observe the effect of both hybridization and triploidization. Vertebrae numbers, scale counts and dorsal fin rays were counted to observe the GDE on the char-salmon hybrid in relation to the parental species. The outcome of this study will help to determine the effect of triploidization and hybridization on morphological expression of meristic characteristics in cross of Atlantic salmon and Arctic char.

Materials and methods

All experiments were carried out at the Institute of Marine Research Matre Research Station in Matre, Norway.

Atlantic salmon incubation temperature experiment

Atlantic salmon ova and milt were acquired from Aquagen (Trondheim, Norway). The eggs from five females were fertilized by a mixture of milt from four males on December 16, 2010. Thirty-seven minutes and 30 seconds after fertilization at 8°C, half of the eggs were subjected to hydrostatic pressure of 655 bar for 6 min and 15 seconds using a TRC-APV Aqua Pressure Vessel (TRC Hydraulics inc., Dieppe, Canada). This produces triploid eggs. Each group of triploid and diploid eggs where then split into 9 replicates. Eggs were then placed into 18 incubation trays in three isolated (UV treatment) flow-through raceways under darkness. The water was buffered with seawater to 0.7 ppt salinity, oxygen saturation of 95% and a pH of 6.9. Each of the raceways contained six incubation trays (3 replicates per ploidy level). Each of the raceways received water with one of three target temperatures of 6°, 8° or 10°C. The actual temperatures varied somewhat during the experiment, but mean temperatures were very close to target temperatures (5.99 ± 0.30°C, 8.00 ± 0.24°C, 9.94 ± 0.22°C; Figure 1). The eggs were mechanically agitated to allow dead eggs to be sorted from live eggs at the eyed-egg stage.

Hatching took place on February 3, 2011 for the salmon incubated at 10°C, February 21 for the 8°C and March 14 for the 6°C (Figure 1). At first feeding (March 7 for 10°C, March 29 for 8°C and May 2 for 6°C) the yolk sack larvae of each incubation tray were then put into single, square grey covered fiberglass tanks (dimensions 1 x 1 m; water depths 30cm). The fish were under continuous light until experiment termination (2 x 18W fluorescent daylight tubes, OSRAM L 18W/840 LUMILUX OSRAM GmbH, Ausburg, Germany). Feeding was continuous using automated disc feeders which started with a commercial start feed (NURTRA ST 0.5, Skretting AS, Fontaine-les-Vervins, France) and increased in pellet size up to 1.5mm (Skretting AS, Norway). During the spring, the water in the tanks where heated up to 13°C until summer solstice (June 21) in which the tanks switched to ambient temperatures (Figure 1).

When the fish reached a body mass of 0.6g (April 7, 2011 for 10°C, April 27 for 8°C and May 31 for 6°C) the number of fish in the diploid tanks were made equal to that of the triploid tanks (~200 fish per tank). This was done to ensure equal rearing conditions between diploid and triploid fish.

The experiment was terminated when fish reached approximately 100g. This target weight was reached at different dates for each incubation temperature (September 19, 2011 for 10°C, October 24, 2011 for 8°C, and January 1, 2012 for 6°C). The fish were euthanized using Finquel® and X-rayed. A total of 90 fish were sampled for each ploidy and incubation temperature for a total of 540 fish.
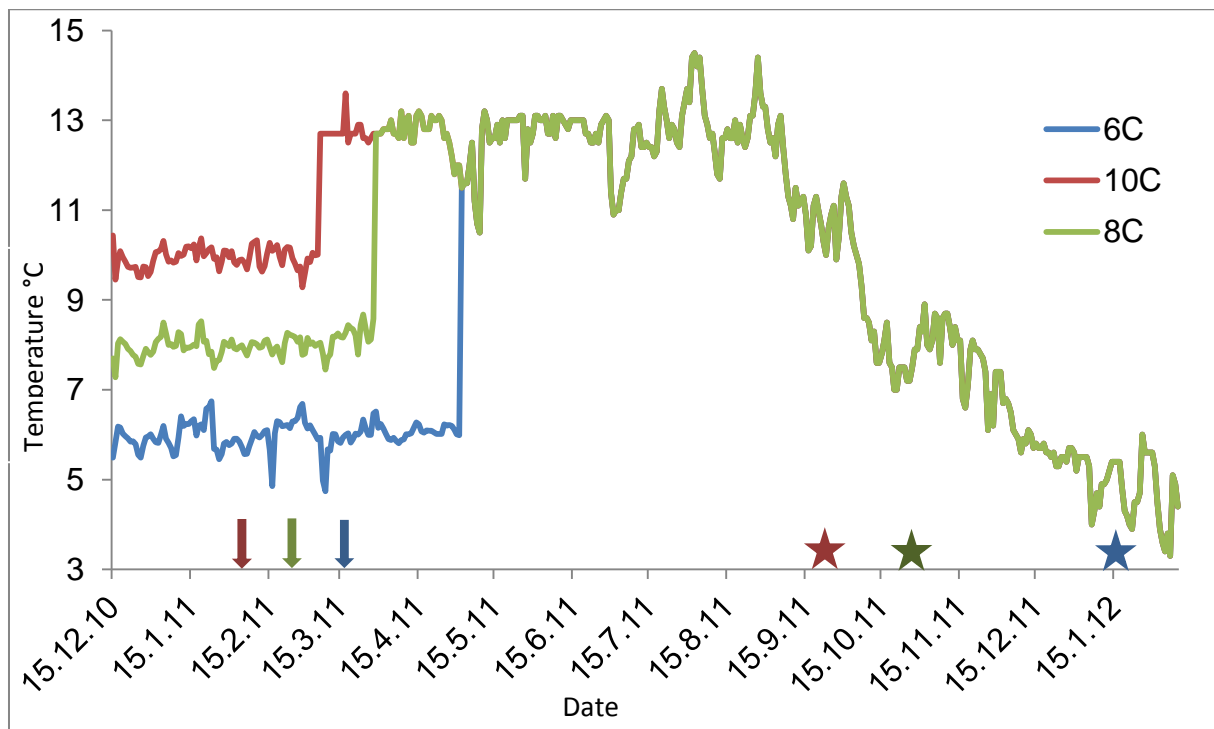

Figure 1: Observed incubation temperatures for each treatment for duration of the Atlantic salmon incubation study. The arrows represent the hatching date for each treatment. Stars represent termination date for each treatment.

Atlantic salmon – Arctic char Hybrid Experiment

On January 1, 2012, eggs and sperm from three male and female Arctic char and Atlantic salmon were used to produce groups of pure Atlantic salmon, pure Arctic char, Atlantic salmon (♀) x Arctic char (♂) hybrids, and Arctic char (♀) x Atlantic salmon (♂) hybrids. The char used were wild caught fish from the lake Skogseidsvatnet in western Norway, and the salmon was of the Aquagen strain. Thirty-seven minutes and 30 seconds after fertilization at 8 °C, half of each group of eggs was subjected to a hydrostatic pressure of 655 bar for 6 min and 15 s (TRC-APV, Aqua Pressure Vessel, TRC Hydraulics inc., Dieppe, Canada), giving eight groups (diploids and triploids of each of the fertilized groups). Each group of eggs was incubated in single incubation trays in a flow-through system at 4.4-8.1 °C under darkness (Figure 2). The eggs were mechanically agitated to allow dead eggs to be sorted from live eggs at the eye-egg stage on February 15, 2012. Hatching took place on March 13, 2012 for both pure Arctic char and char-salmon hybrid and on March 23, 2012 for Atlantic salmon.

On May 2 2012, the fry of each incubation treatment was randomly distributed between three covered fiberglass tanks (1×1×0.35 m) and placed under continuous light and at 12.4 °C. There were no surviving larvae from the diploid and triploid Arctic char (♀) x Atlantic salmon (♂) hybrid groups at first feeding. The photoperiod in the tank period was 24 h continuous light, and the temperature ranged between 11.2 and 13.5^{o}C up until 28 August 2012 and then decreased gradually with ambient temperatures until October 3, 2012 (Figure 2). Feeding was continuous using automated feeders and fish were fed in access. Fish started feeding with a commercial start feed (NURTR XP 0.3mm, Skretting AS, Norway) and increased in pellet size according to fish size.

On October 3, 2012 the experiment was terminated. Fish were euthanized using an overdose of Finquel®, photographed and X-rayed for analysis. Only 8 triploid Arctic char and 19 triploid char-salmon hybrids survived to experiment termination. Therefore, 19 fish were sampled for each group (8 for the pure Arctic char) making a total of 309 fish sampled.
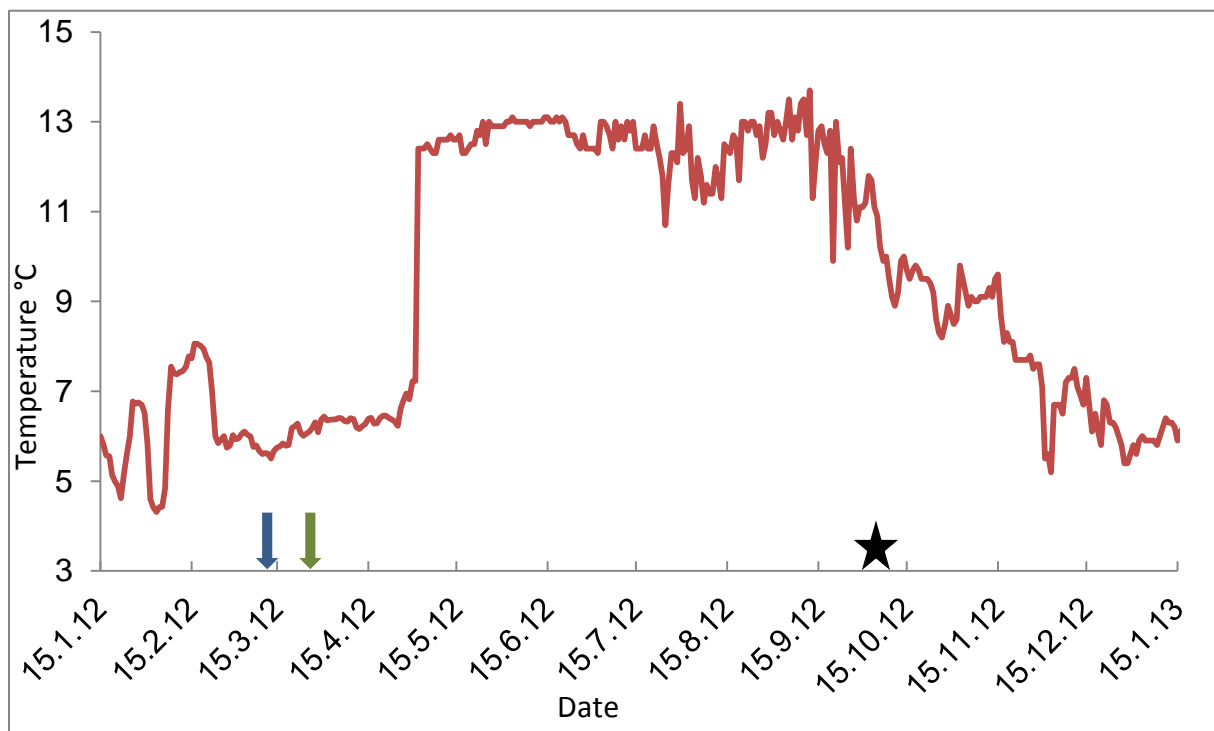
Figure 2: Observed water temperatures for Atlantic salmon Arctic char hybrid experiment. Incubation temperatures varied 4.4°C-8.1°C. After transfer to tanks at 13°C water temperature decreased with ambient temperatures. Arrows indicate hatching (blue: Arctic char & char-salmon hybrid; green: Atlantic salmon). Star indicates termination of experiment.

Ploidy verification

For both experiments, during sampling, blood was drawn using a syringe which was inserted vertically into the dorsal end of the fish between the anal fin and tail fin. Small samples were taken (~1mL) and blood smears were made. Measurements of erythrocyte diameter were used to verify ploidy status. 80 fish from each ploidy was sampled for the Atlantic salmon incubation study and 10 fish from each group (8 from char group) was measured for the hybrid experiment. 10 erythrocytes per fish was measure (Image-Pro Plus, version 4.0, Media Cybernetics Silver Spring). There was no overlap in mean erythrocyte diameter between diploid and triploid individuals (Table 1), suggesting 100% efficiency of the triploidy induction (Figure 3).
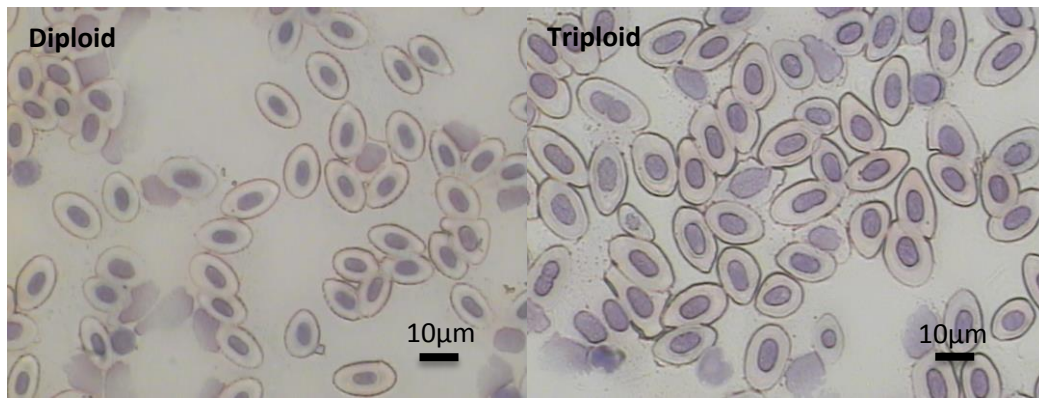
Figure 3: Blood smears of diploid and triploid erythrocytes. Larger erythrocytes indicate triploid (Photo: Migaud H)

Table 1: Erythrocyte diameter use for ploidy confirmation. a.) Mean diameter (± sd) of diploid and triploid Atlantic salmon. b.) Mean diameters (± sd) of diploid and triploid pure Arctic char, Char-salmon hybrid and pure Atlantic salmon erythrocytes.

a).

Specie	Erythrocyte diameter (µm)		
	N	Diploid	Triploid
Atlantic salmon	80	16.9 ± 1.8	21.4 ± 1.1

b.)

Specie	Erythrocyte diameter (µm)		
	N	Diploid	Triploid
Arctic char	8	18.6 ± 1.7	22.4 ± 2.2
Char-salmon hybrid	10	18.8 ± 1.7	22.1 ± 1.2
Atlantic salmon	10	17.1 ± 0.8	20.9 ± 1.1

Sampling protocol

All fish in both experiments were killed by an overdose of anesthetic (Finquel®) and X-rayed (Porta 100 HF; Eickemeyer Medizintechnik für Tierärzte KG, Tuttlingen, Germany). The settings of the X-ray machine were set to 40kV and 10 mAs and X-rays were taken from a distance of 70cm. The image plate used was 35x43cm (Dürr Medical, Bietigheim-Bissingen, Germany) and was scanned using the CR 35 VET scanner (Dürr Medical Bietigheim-Bissingen,

Germany). All vertebrae were counted using Image J (Rasband 2012) counting the most dorsal vertebra as vertebra 1.

Vertebra deformities were determined using a deformity key for salmonids developed by Witten et al (2009). The main deformities that were identified were types 5-9 (Appendix 1). If complete fusion occurred, the number of vertebra was counted via the number of neural spines (Figure 4). Due to fusion events being too severe to determine number of neural spines a total of 20 fish were removed from the sample pool (Table 2). Vertebra size estimated as vertebrae area from the X-ray pictures was determined using Image J polygon tool marking each corner of the vertebra and measuring the area inside.

Table 2: Number of fish sampled of each ploidy for vertebrae number and deformity prevalence at each temperature treatment for the Atlantic salmon incubation temperature experiment.

Incubation temperature	Vertebra count		Deformity prevelence	
	Diploid	**Triploid**	**Diploid**	**Triploid**
6°C	90	89	30	30
8°C	90	87	30	30
10°C	83	81	30	30
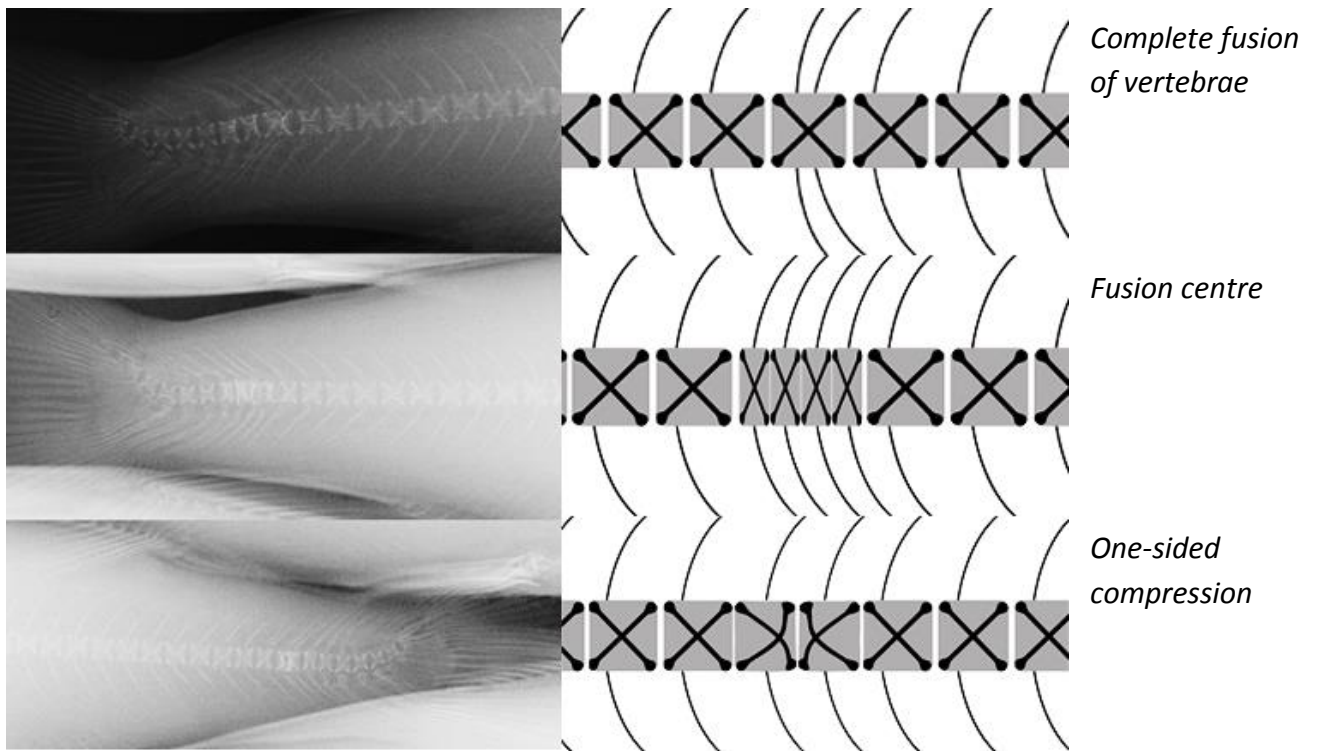
Figure 3: Examples of most common deformities observed in Atlantic salmon incubation study
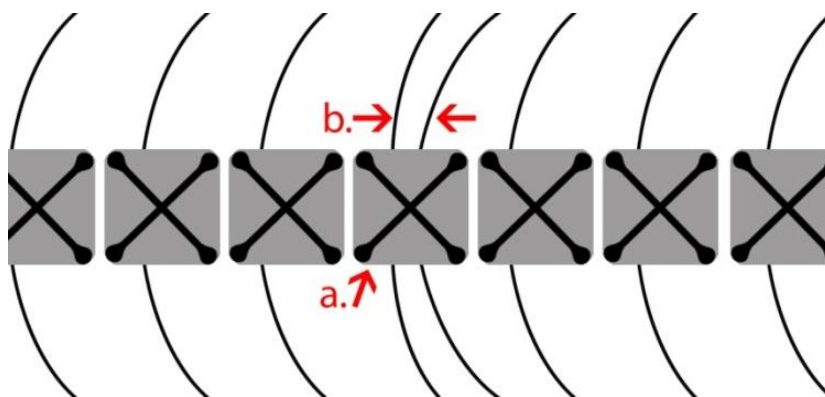
Figure 4: Diagram representing a complete fusion deformity in Atlantic salmon. If this deformity was present (a.) the number of vertebra fused would be determined by counting the number of neural spines (b.)

For the salmon char hybrid experiment the number of scales and dorsal fin rays were counted using high resolution photos (Sony Nex5) which were imported into Image J (Table 3). The numbers of scales were counted along the lateral line of each fish. Vertebra numbers were counted using X-rays and if severe deformities were present fish were removed from

the sample pool. Due to low survival of triploid char only 8 were available for sampling. 19 fish were sampled for each of the other groups. Scaled counts had lower sampling numbers due to quality of photos not allowing for accurate counts.

Table 3: Number of fish sampled for each of the various meristic characteristics analyzed in the char salmon hybrid experiment

| Parameter | Artic char | | Char-salmon | | Atlantic salmon | |
	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid
Vertebra count	19	8	14	18	19	19
Scale count	12	5	10	10	10	10
Dorsal fin rays	19	8	19	19	19	19

Statistical analysis

All statistical analysis was done using Statistica 10 (Stats soft inc, OK USA). To test the effect of ploidy and incubation temperature on vertebral numbers I used ordinal logistic regression. This model was used because vertebra number is non-continuous and not normally distributed. The model was structured with vertebral counts as the dependent variable with temperature and ploidy as categorical independent variables.

For the salmon incubation study the mean area for each vertebra was graphed versus vertebra number (position along the vertebrae) for each ploidy and incubation temperature. The difference in area size between ploidy groups was standardized equation 1. A *t*-test was then implemented to determine area difference between incubation temperatures. Each incubation temperature was treated as an independent variable and tested against each other.

$$\gamma = \left[\frac{(\bar{x}n - \bar{y}n)}{(\overline{xy})} \right] i \qquad\qquad Equation\ 1$$

For the salmon char hybrid experiment Mann-Whitney U tests were used to test for differences in the meristic characteristics between ploidy. Mann-Whitney U test were used because of the greater detail it provides when data is not normally distributed.

Ethical considerations

These experiments followed general regulations for animal experimentation. Precautions were taken to provide the fish with comfortable environments which provided all necessary needs. Fish were inspected each day but making sure to keep disturbances to a minimum.

Results

Atlantic salmon incubation temperature treatment experiment

The observed mean values for weight of each fish group varied slightly from the target weight of 100g (Table 4). The mean values of weight ranged from 83.4g in diploid 8°C to 106g in triploid 10°C (Table 4). There was large range of weights and lengths observed in all treatments of the experiment. The smallest fish measured was 12g at 9.5cm long and the largest fish was 161g at 22.5cm long, both these fish were triploid and incubated at 8°C. In general triploid fish were larger and heavier than diploids (Table 4).

The mean number of vertebrae in Atlantic salmon varied between 57.6 for triploid fish at 10°C and 58.6 for diploid fish at 6°C (Table 4). The maximum number of vertebra observed was 60 and the minimum was 56. In general, vertebral counts were lower in triploid than in diploid salmon at all three incubation temperatures (Table 4, 5). However, the overall effect size was small, with a reduction in mean vertebra count of from 0.4 to 0.8 vertebrae. Further, there was a general trend that vertebrae counts decreased with increasing incubation temperature (Table 4, 5). Ordinal logistic regression displayed a statistically strong difference between the reaction norms of diploid and triploid salmon; in that increasing incubation temperatures induced a more severe decline in vertebra number in triploids than diploids ($P < 0.05$, Table 5).

The total number of deformities in each fish were counted and analyzed for deformity prevalence with relation to incubation temperature and ploidy. The most common type of deformity seen in this study was one-sided compression, compression and fusion, complete fusion, fusion centre and elongation (Types 5-9 according to key by Witten et al., 2009; Appendix 1). The highest number of deformities in a single fish was 15 and was present in a 10°C triploid fish. In general, the fish incubated at 6°C had low numbers of deformities with a large proportion of sampled fish having no deformities at all (Table 6). The number of deformed vertebrae as well as the prevalence of deformities increased with increasing temperature. In addition, more deformities were seen in triploid fish at all three incubation temperatures (Table 6).

The size (area) of each vertebra was measure to determine if there is an effect of ploidy and/or incubation temperature. The area of each vertebra differed according to the position along the body. The vertebra close to the head and tail were the smallest, reaching largest area around vertebra no. 38-42 (Figure 4). This pattern was a general trend seen at all incubation temperatures and for both diploid and triploid fish (Figure 4). The area of each vertebra was larger in triploids than diploids for the 6° and 8°C treatments (Figure 4). This observation reversed for the 10°C treatment with the vertebra size in diploids being larger than triploids (Figure 4). The fish incubated at 6°C had the largest difference in vertebra size (Figure 4).

To show statistical evidence of this size difference equation 1 was used to standardize the difference in observed areas of each vertebra. A standardized value of 0 represents no difference in vertebra areas between diploid and triploid salmon. The mean values of equation 1 decreased from 0.154 for 6°C to -0.015 for 10°C. In all cases the standardized values were significantly different than zero confirming vertebra size (area) difference between diploids and triploids (Table 7).

Table 4: Mean (±sd) length, weight and vertebra numbers for diploid and triploid Atlantic salmon incubated at various incubation temperatures.

Incubation temperature	Parameter	Diploid	Triploid
6°C	Length (cm)	18.5±1.2	20.5±1.8
	Weight (g)	89.1±17.6	103.7±23.3
	Vertebra number	58.6±0.1	58.2±0.1
8°C	Length (cm)	18.3±1.8	19.2±2.0
	Weight (g)	83.4±21.7	98.0±26.4
	Vertebra number	58.5±0.1	58.1±0.1
10°C	Length (cm)	19.2±1.7	19.7±1.6
	Weight (g)	95.0±15.2	106.1±20.1
	Vertebra number	58.4±0.1	57.6±0.1

Table 5: The difference vertebra number according to temperature and ploidy. Difference in thermal growth reaction norms between diploids and triploids are represented by Ploidy*Temerature. Test was done using an Ordinal logistic regression model.

Effect	Degree of Freedom	Wald Stat.	P value
Intercept	4	426.21	0.000
Temperature	2	36.34	0.000
Ploidy	1	95.60	0.000
Ploidy*Temperature	2	7.67	0.026

Table 6: The percent of sampled fish who had 1 or more deformed vertebra (N=30) and observed range of deformed vertebra for diploid and triploid salmon at each incubation temperature.

Incubation Temperature	Parameter	Diploid	Triploid
6°C	% Deformed	13	30
	Range	1-2	1-5
8°C	% Deformed	33	50
	Range	1-5	1-6
10°C	% Deformed	57	93
	Range	1-8	1-15
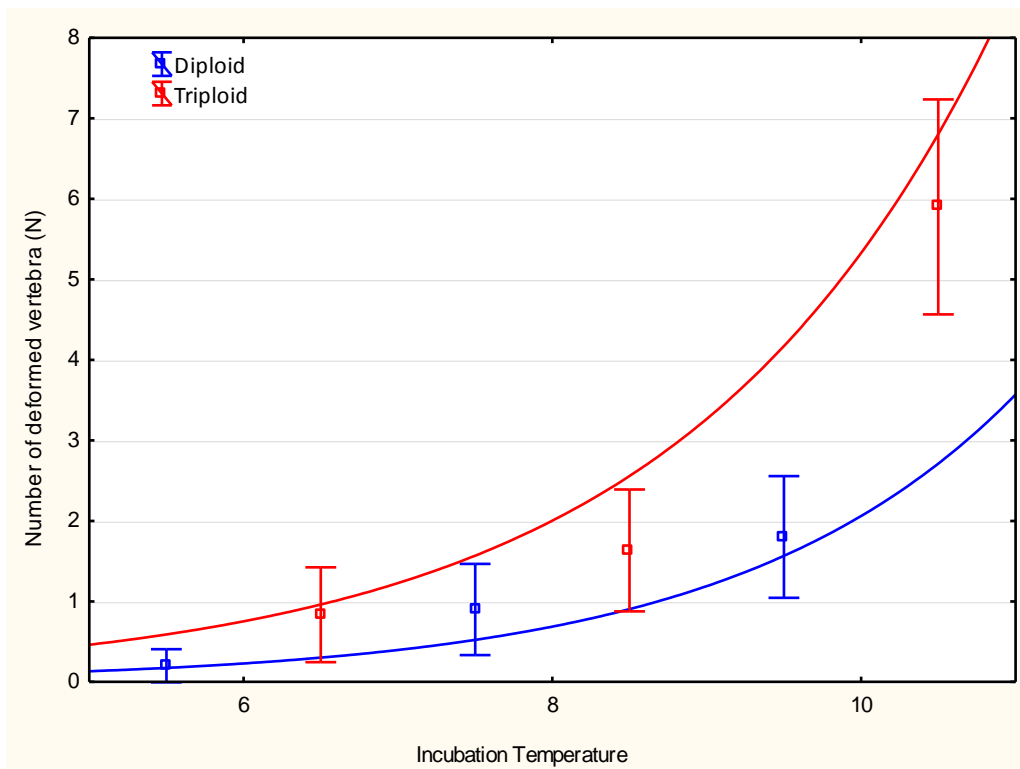
Figure 3: The average number of deformed vertebra (mean ± 95%) observed in diploid and triploid Atlantic salmon incubated at different incubation temperatures (6°C, 8°C and 10°C). Trend lines represent best fit lines for each polidy.
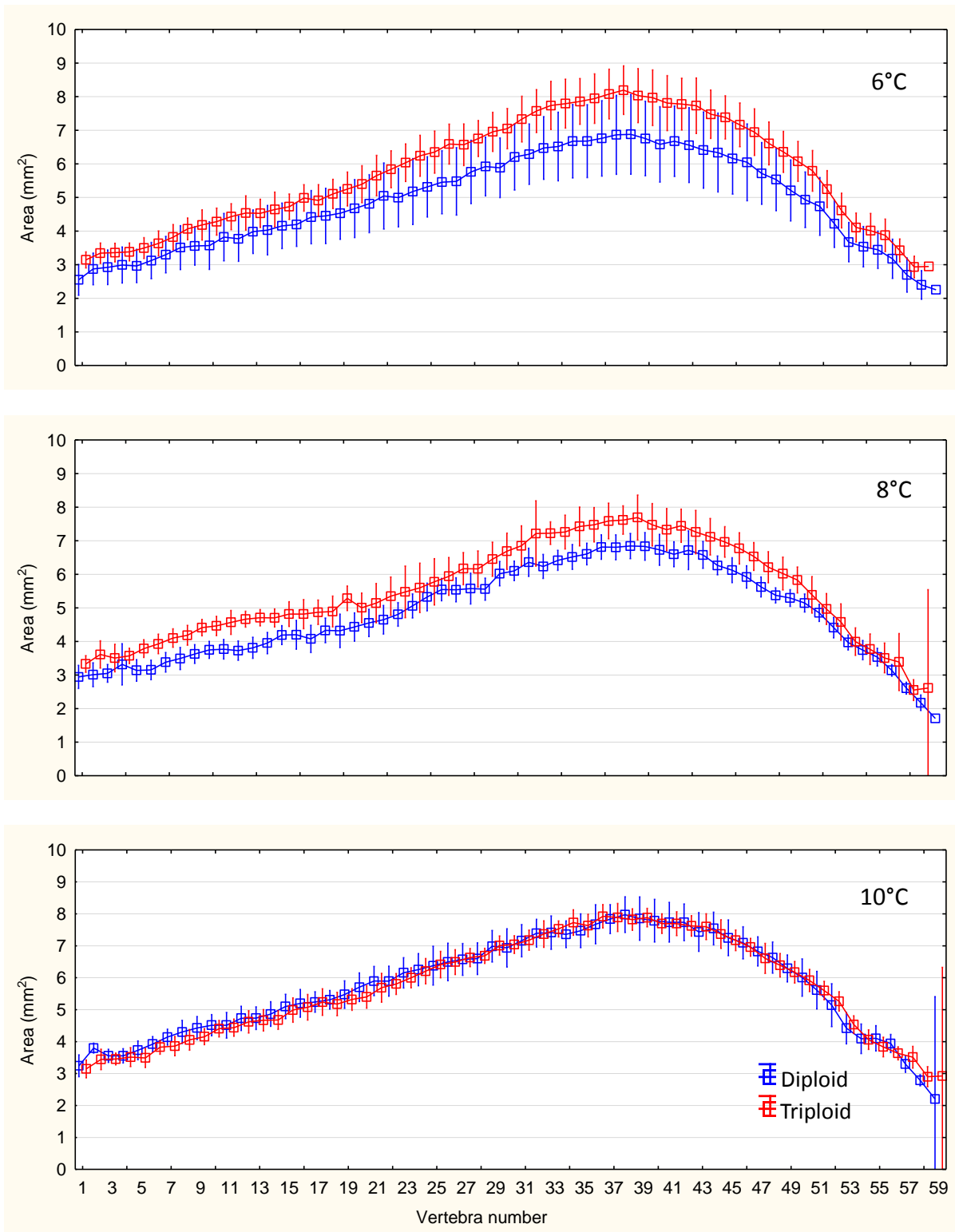
Figure 4: Graphs showing mean value (± 95%) of each vertebra along the vertebra column of diploids and triploids for each temperature treatment (6°C, 8°C, and 10°C) for the Atlantic salmon incubation study.

Table 7: *t*-test testing single samples (incubation temperatures) to a reference constant (0.00)

Incubation temperature	Mean	Std.Err.	t-value	df	P value
10°C	-0.015	0.004	-3.72	56	<0.001
8°C	0.120	0.007	17.28	56	<0.001
6°C	0.154	0.003	44.44	56	<0.001

Atlantic salmon – Arctic char Hybrid Experiment

There was a large range in size for most groups but in particular the diploid Char-salmon hybrids. The smallest diploid hybrid measured as 8.8 cm weighing 7g and the largest was 19.1cm weighing 92g. The smallest fish in general were the triploid char with a mean size was 8.2 cm weighing in at just under 9g; only 8 triploid pure Arctic char survived till sampling (Table 8). The largest fish were the Atlantic salmon with a mean value of 68.9g and 17.1cm long for diploids and 65.4g and 17.1 cm long.

The vertebrae numbers for the pure Atlantic salmon, pure Arctic char and Char-salmon hybrid were counted (Table 9). The mean number of vertebra for the hybrid experiment varied from 58.3 in the triploid Atlantic salmon to 59.9 in the triploid Arctic char. The vertebra numbers of diploid and triploid Arctic char did not differ (Table 9). The diploid Char-salmon hybrid had a vertebra count of 59.0 which was intermediate to the parental species however the triploid hybrid had vertebra numbers similar to the triploid Arctic char (Table 9). Both the triploid Arctic char and triploid Char-salmon hybrid had higher vertebra counts than the diploids, different to that of the triploid Atlantic salmon whom had lower vertebra numbers.

The number of scales each fish had was determined by counting scale rows along the lateral line. Scale numbers varied from 195 in the diploid Arctic char to 135 in the triploid Atlantic salmon (Table 10). The char-salmon species had intermediate counts to the parental species of 153 and 145 for diploid and triploid respectively. There was a general trend of lower scale counts in triploids than in diploids for all three groups.

The numbers of dorsal fin rays were lower in triploids than diploids in both the pure Arctic char and the char-salmon hybrid (Table 11). The diploid Atlantic salmon had higher dorsal fin rays than the triploids. Dorsal fin ray counts varied from 11.3 in the triploid Arctic char to 14.1 in the Diploid Atlantic salmon. No significance in dorsal fin ray count was observed between ploidy of the Atlantic salmon, both having roughly 14 fin rays. The char-salmon hybrid, in general, had intermediate numbers of fin rays in relation to the parental species (Table 11).

Table 8: Mean (±sd) lengths and weights for each group of the Atlantic salmon Arctic char experiment.

Parameter	Arctic Char		Char-Salmon		Atlantic Salmon	
	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid
Length (cm)	13.2 ± 1.3	8.2 ± 0,6	12.6 ± 2.8	54.6 ± 16.9	17.1 ± 0.9	17.2 ± 0.6
Weight (g)	27.7 ± 9.1	8.8 ± 2.0	27.6 ± 21	16.4 ± 1.7	68.9 ± 10.0	65.4 ± 7.3

Table 9: Mean (±sd) vertebra numbers of diploid and triploid Arctic char, char-salmon hybrid and Atlantic salmon. Mann-Whitney nonparametric test used to observe difference of vertebra number between ploidys.

Specie	Vertebra number		Rank Sum		P value
	Diploid	Triploid	Diploid	Triploid	
Arctic char	59.7 ± 0.2	59.9 ± 0.2	61.0	75.0	>0.05
Char-salmon	59.0 ± 0.2	59.9 ± 0.2	161.5	366.5	0.006
Atlantic salmon	58.6 ± 0.2	58.3 ± 0.1	410.5	330.5	>0.05

Table 10: Mean (±sd) number of scales in diploid and triploid Arctic char, char-salmon hybrid and Atlantic salmon. Mann-Whitney nonparametric test used to observe difference of scale counts between ploidys.

Specie	Scale count		Rank sum		P value
	Diploid	Triploid	Diploid	Triploid	
Arctic char	195 ± 2	191 ± 2	117.0	36.0	>0.05
Char-salmon	153 ± 2	145 ± 1	139.5	70.5	0.010
Atlantic salmon	141 ± 1	135 ± 1	145.5	9.5	0.002

Table 11: Mean (±sd) dorsal fin ray count in diploid and triploid Arctic char, char-salmon hybrid and Atlantic salmon. Mann-Whitney nonparametric tests used to observe difference of dorsal fin rays between ploidys.

Specie	Dorsal fin ray count		Rank sum		P value
	Diploid	Triploid	Diploid	Triploid	
Arctic char	12.0 ± 0.1	11.3 ± 0.3	303.0	75.0	>0.05
Char-salmon	13.5 ± 0.2	12.7 ± 0.1	468.0	273.0	0.002
Atlantic salmon	14.1 ± 0.1	14.0 ± 0.1	396.0	345.0	>0.05

Discussion

The results of this experiment displayed a strong effect of triploidization on Atlantic salmon and Atlantic salmon hybrids. Triploidization along with increasing incubation temperatures caused decreased vertebra number in triploid Atlantic salmon. In addition, the number of deformities and deformity prevalence were higher in triploid salmon and were strongly influenced by increasing incubation temperature. With regards to the meristic characteristics of the char-salmon hybrid, expression was mosaic; meaning that each feature investigated has different levels of resemblance to either parental species. Certain characteristics of the hybrids such as length, weight and vertebra number were more similar to the pure Arctic char whereas scale counts were more similar to the pure Atlantic salmon.

Atlantic salmon Incubation temperature experiment

This study observed decreased vertebra number and increased deformity prevalence in Atlantic salmon as a result of triploidization and increased incubation temperature. An inverse relationship between incubation temperature and vertebra number was seen in both diploid and triploid fish. Vertebra size (area) analysis revealed that at low incubation temperatures vertebra were smaller in triploids than in diploids. This trend switched at 10°C where the vertebra of diploids alternated to being larger than triploids. When investigating the response of vertebra number to different incubation temperatures it was found that diploid and triploid salmon have different reaction norms. This indicates that triploid salmon may require different farming regimes than diploids in order to keep vertebra number at appropriate levels and keep deformity prevalence low.

High incubation temperatures have been known to reduce incubation period in teleost fish (Pepin et al., 1997; Crisp 1981). Incubation period is related to life history traits such that it is synchronized with suitable environmental factors to increase things such as food availability and survival (Huey and Kingsolver 1989). However, with regards to the farming industry reduced incubation period means earlier exogenous feeding which allows for quicker transfer to seawater where growth is highly accelerated (Usher et al., 1991; Fjelldal and Hansen 2006). Therefore increased incubation temperatures are used within farming

regimes in order to speed up incubation period, yet the results of this study show it may have counterproductive results by decreasing vertebra numbers and increased deformities.

With regards to both diploids and triploids, a significant decrease in vertebra number with increasing incubation temperature was observed. In addition, triploid salmon had lower vertebra numbers than diploids at all incubation temperatures. Low vertebra numbers at high incubation temperatures has been shown in many studies (Fowler 1970; Ando et al., 2011; Finstad and Jonsson 2012) yet my study is the first which shows this trend being amplified by inducing triploidy. Triploidization results in the acquisition of a third set of chromosomes and therefore a significant increase of genetic material. Therefore it could be that by increasing the quantity of genetic material you also increase the sensitivity to abiotic factors.

The effect of water temperature during embryonic development has been suggested to be the most crucial factor with regards to development timing and accuracy (Tåning 1950; Fowler 1970; Jonsson & Jonsson 2012). Meristic characteristics vary slightly within populations of teleost fish and this natural variation has a tight correlation to abiotic temperatures (Barlow 1961; Wilkens et al., 2010). Things such as fin rays, scale number and skull morphology will be decided after hatching but the final number of vertebra a fish will have is determined early in embryonic development by somite formation (Fowler 1970). Tåning (1950) termed this period of embryonic development a "super sensitive period" where slight variations in temperature can have a dramatic effect on the final number of vertebra. Fowler (1970) discussed the possible evolutionary aspects behind such variation seen in populations. He mentions that if such slight variation in vertebra number has no selective advantage then this variation will continue. This is true, as variations in vertebra number has been mentioned within population of fish with no real relationship to fitness (Ando et al., 2011). This, however, is in natural populations and the results of this study show that even slight variation in vertebra number is tightly correlated to deformity prevalence in fish reared in farming conditions, this topic will be discussed later on.

There was an observed difference in the thermal reaction norms between diploid and triploid fish. Both ploidys displayed a negative reaction norm with vertebra number progressively decreasing with increasing water temperature; however, the reduction in

vertebra number was more severe for triploids than diploids. Reaction norms are largely influenced by genetics and are the phenotypic response to abiotic factors. Different populations display different reaction norms and thus suggest that there are different optimal growth patterns for different abiotic conditions. An example of this is in Pacific salmon (*Oncorhynchus* spp.) in which display both V-shaped and negative reaction norms depending on where they are from (Ando et al., 2011). A less severe example relates slight genetic changes to changes in reaction norms which has the potential to have a direct effect on the fitness of Atlantic salmon (Darwish & Hutchings 2009). The different reaction norms observed in this experiment suggests different optimal growth conditions for triploid salmon in farm reared conditions. Thus, in agreement with Benfey (1999), triploid salmon should be treated different than diploid salmon when considering rearing conditions for aquaculture use.

When discussing possible mechanisms behind the decreased vertebra number with increasing incubation temperature, and the difference in vertebra number between ploidys, it is important to remember that the effect size was small. The overall difference averaged to less than 1 vertebra however this suggests that a single vertebra may be the difference between a deformed or non-deformed fish. Thus, although a small difference in vertebra number between diploid and triploid salmon it may be important to find optimal temperatures for triploid fish to develop in a way that maintains vertebra number at appropriate levels and keep deformity prevalence low.

The main types of deformities observed in this experiment were one-sided compression, compression and fusion, complete fusion, fusion centre and elongation (types 5-9 Appendix 1; Witten et al., 2009). At all incubation temperatures there was a higher occurrence of deformities in triploid than in diploid salmon and effected by far were the triploid fish incubated at 10°C (the highest temperature used). The relationship between incubation temperature and vertebrae deformities have been mentioned before in studies done on Atlantic salmon (Witten et al., 2005; Fraser et al., 2013) yet this relationship in triploids has yet to be elucidated. Mechanical load on the vertebral column, gene expression and nutrient transport to and from the notochord during development have been suggests as possible pathways of deformity development.

As mentioned earlier, domesticated salmon have been selectively bred for generations to acquire traits that would be advantageous to farming. One of these traits is increased muscle growth which in turn provides more meat and higher production value for the industry. However, increased muscle growth can cause increased mechanical load on notochord cells during early vertebra development (Lotz et al., 2003). Pressure via mechanical stress on the notochord cells initiate the conversion of the notochord and the notochord sheath into cartilage which eventually mineralizes into bone during vertebral column development (Witten et al., 2005; Nordvik et al., 2005). The mechanical stress of the increased muscle mass can cause altered growth which in turn may lead to altered vertebra shape (Witten et al., 2005; Yttebor et al., 2010b). If pressure is caused from increased muscles mass then larger cells sizes due to the acquisition of a third set of chromosomes may cause increased pressure on notochord cells thus increasing the severity of altered vertebra development.

Atlantic salmon eggs incubated at high and low temperatures were investigated for gene expression levels by Ytteborg et al (2010). In doing so, at high incubation temperatures, Ytteborg observed high expression levels of transcription factors which disrupt the accuracy of notochord segmentation (Yttebor et al, 2010a, b). The notochord segmentation is the precursor to vertebrae formation (Grotmol et al., 2005; Nordvik et al., 2005) and thus disrupting this process may lead to increased deformities. In addition, if increased genetic material (by triploidization) results in increased genetic expression it may be that triploid fish have increased inhibitory gene expression levels. In a similar fashion, it has been shown that haploid eukaryotes display decreased levels of regulator genes when compared to diploids (Birchler et al., 2005). Therefore it may be that triploidization causes higher vertebral deformities by increased expression of inhibitory genes yet a detailed analysis of expression levels would be needed in triploids in order to make concluding remarks.

The higher prevalence of fusion events seen in triploids may be due to reduced effectiveness of the notochord sheath functionality. Ytteborg et al. (2010c) suggested that the notochord sheath assists in nutrient and waist transport to and from the notochord during early embryonic stages. In addition, Parsons et al. (2002) suggested that in teleost fish the notochord sheath helps maintain hydrostatic pressure within the notochord. Both of these functions play and important role in proper notochord development and if disrupted could lead to a higher occurrence of fusion events (Ytteborg et al., 2010c). As mentioned earlier, a

repercussion of triploidization is larger cells and thus a reduced surface area to volume ratio. This physiological effect is suggested to reduce overall nutrient transport in triploid fish (Maxime 2008; Tiwary et al., 2004). Therefore, the physiological side effects of triploidization may further reduce the functionality of the notochord sheath; in turn disrupting proper notochord development leading to higher fusion prevalence.

These explanations offer possible areas of development that may be affected by triploidization. However, it is safe to assume that the higher occurrence of vertebra deformities observed in this study is not simply due to one mechanism but rather a combination of many. Therefore it is mostly likely a combination of these mechanisms, and possible many more that contribute to the higher occurrence of deformities seen in triploid salmon.

Observed in this study was a difference in vertebra size (individual vertebra along the vertebra column) between ploidys at both 6° and 8°C with triploids being smaller than diploids. At 10°C however, the difference in size between ploids was relatively small with diploids being slightly larger than triploids. The trend of varying vertebra size along the vertebral column has been acknowledged before in Atlantic salmon (Fjelldal et al., 2005) yet this is the first time a difference in size between ploidys has been mentioned. The explanation for this observation is hard to determine due to lack of supporting evidence; yet considering there was an observed difference in thermal reaction norms for vertebra number, it may be hypothesized that there is a difference in thermal reaction norms for vertebra size between diploid and triploid Atlantic salmon.

This study displays a significant effect of ploidy and incubation temperature on vertebra number, size and deformity prevalence in Atlantic salmon. Vertebra numbers are determined during a "super sensitive" period of development and small fluctuations in temperature can affect the final number (Tåning 1946). High incubation temperatures may have an indirect connection to vertebra number via gene expression and nutrient transport affecting notochord development. In addition, deformities may be more prevalent in triploids due to increased genetic material causing both increased gene expression and decreased nutrient transport. The results of my study suggest different thermal reaction norms for both vertebra number and vertebra size for diploid and triploid fish, yet

experiments testing a wider range of incubation temperatures would give a deeper incite to triploid reaction norms. The combined results suggest that new farming regimes should be used when farming triploid Atlantic salmon in order to maintain appropriate vertebra number and low deformity prevalence.

Atlantic salmon-Arctic char hybrid experiment

In this study I investigated the morphological expression of vertebra number, scale count and dorsal fin rays from a cross between Atlantic salmon and Arctic char. The effort was to understand how triploidization affects phenotypic expression since triploidization effects the genetic contribution from the parental species. Interspecific hybridization has been studied in salmonids for many years (Bartley et al, 2001; Gray et al., 1993; Scheerer and Thorgaard 1983; Refstie and Gjedrem 1975). Interspecific hybridization is the breeding of closely related species in hopes of creating a viable cross that out perform their parental species in certain aspect.

Arctic char and Atlantic salmon crosses have shown to produce viable offspring with good hatching and growth rate (Refstie and Gjedrem 1975). However, in the present experiment there were no viable offspring from the cross between male Arctic char and female Atlantic salmon. Gray et al (1993) found that the viability of hybrids is related to the hatching time of the female species. In that, more viable progeny are produced when crossing a female species with a faster developmental rate than the male species. In this experiment the Atlantic salmon were of the Aquagen strain in which generations of breeding have most likely selected for fast development. Therefore, it may be that no viable offspring were created when crossing the fast growing male salmon with the wild caught and probably slow-growing female char.

An effect of ploidy and hybridization was observed in all three meristic characteristics I investigated in this study. With regards to ploidy, scale counts and dorsal fin ray counts were lower in the triploid fish than in diploids as well as the vertebra numbers for Atlantic salmon. However, the triploid char and char-salmon hybrid had higher vertebra numbers than diploids. Similarly, both diploid and triploid char-salmon hybrid had mean vertebra numbers

similar to that of the Arctic char. On the contrary, the number of scales and dorsal fin rays were intermediate to that of the parental species. This difference in expression between meristic characteristics was unexpected if phenotypic expression is linked to genomic contribution.

The Atlantic salmon genome consists of 58 Arctic whereas the char's has 80 chromosomes (Hartley 1987). The char-salmon hybrid in this experiment was created using a female Atlantic salmon and male Arctic char thus consisting of 29 salmon and 40 char chromosomes. When triploidy is induced the maternal contribution is doubled. Therefore the triploid Char-salmon hybrid investigated in this experiment consisted of 58 (2 x 29) chromosomes from the Atlantic salmon and 40 from the Arctic char making a total of 98 chromosomes. In plants, there is a linear relationship between genomic contribution and phenotypic expression thus meristic characteristics of hybrids are more similar to that of the higher genomic contributing parental species (Riddle et al., 2010; Chen & Ni, 2006). However, the results of my experiment display a mosaic pattern of phenotypic expression with different meristic characteristics leaning towards different parental species.   This mosaic style patterning has been observed before in other hybrids including grass carp (*Ctenopharyngodon idella*), water frogs (*Pelophylax shqipericus*) and fire-bellied toads (*Bombina bombina*)(Cassani & Caton 1984, Vörös et a., 2007; Keirzkowski et al., 2011).

Predicting traits of hybrid fish could be a huge advantage towards discovering new species of fish for domestication. That being said this study shows the difficulty in doing so. Varying degrees of expression with regards to the parental species suggests a nonlinear relationship between phenotypic expression and genetic contribution. However, this experiment did show that certain traits of a hybrid species may resemble one parental species far more than the other. Vertebra numbers of the Char-salmon hybrid were nearly identical to that of the Arcitc char. This suggests that it may be possible to select for desired traits, yet a better understanding of the mechanisms that control this is needed.

Improvements

They X-ray machine used in this experiment was great for sampling large number of fish and X-raying them with high enough resolution for vertebra counts and deformity recognition. It did however lack the resolution to do a detailed analysis of vertebra deformities (to include minor deformities such as misalignment) and the ability to determine how many vertebras were lost when severe fusion events occurred. Therefore, if a more detailed analysis of vertebra deformities were to be done a higher resolution X-ray machine would be needed.

Conclusion

The results of my study suggest an answer for the first goal of this study in determining better rearing conditions for triploid Atlantic salmon. At all incubation temperatures tested in this experiment triploid salmon had less vertebra numbers than diploids. The trend of decreasing vertebra numbers with increasing incubation temperature was present in both diploid and triploid salmon but the decrease in vertebra numbers was more severe for triploids. In addition, the number of deformed vertebra dramatically increased with increasing incubation temperature. Therefore lowering the incubation temperature for triploid fish should be taken into consideration when rearing conditions are determined for triploid fish in order to reduce the presence of deformities and keep vertebra numbers at appropriate levels.

Part two of my study raised awareness to the complexity of hybrid morphology. The goal was to determine if morphological features of triploid hybrids could be predicted due to genetic contribution from parental species. I investigated vertebra number, scale counts and dorsal fin rays and found no general linear relationship between genetic contribution and phenotypic expression, at least for Atlantic salmon x Arctic char hybrid. Therefore this study reveals that predicting morphological traits of triploid hybrids via genetic dosage may be impossible or at least until a better understanding of the genetic dosage effect is understood in salmonids.

Investigating more features of triploid hybrids may allow for a better understanding of how meristic characteristics are expressed. Having a library of features and how they relate to parental species may reveal a general pattern which is not visible from few features I investigated in my study.

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